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VRIJBLOED, JW; MADON, J; DIJKHUIZEN, L

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A Plasmid from the Methylotrophic Actinomycete *Amycolatopsis methanolica* Capable of Site-Specific Integration

J. W. VRIJBLOED,[1] J. MADOŃ,[2]† AND L. DIJKHUIZEN[1]*

Department of Microbiology, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands,[1] and Mikrobiologisches Institute, ETH-Zentrum, Universitätsstraße 2, CH-8092 Zürich, Switzerland[2]

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***Amycolatopsis methanolica* contains a 13.3-kb plasmid (pMEA300) which is present both in the free state and integrated at a unique genomic location. A 2.1-kb pMEA300 DNA fragment was sequenced, revealing the putative *attP* site and two open reading frames, *xis* and *int*, showing similarity to genes encoding excisionases and integrases, respectively.**

Actinomycetes are gram-positive bacteria that form elongated, branching cells and produce the majority of the currently known secondary metabolites. Genetic studies with various actinomycetes have resulted in the identification of self-transmissible DNA elements present both in the free state and chromosomally integrated (5, 8, 10, 15, 18, 19, 21, 25). For some of these elements (6, 10, 17, 20, 25), the processes of site-specific excision and integration have been shown to involve recombination between attachment sites on the plasmid (*attP*) and on the bacterial chromosome (*attB*). We are interested in the physiological role of such an integrating plasmid (pMEA300) in *Amycolatopsis methanolica* NCIB 11946 (11). This nocardioform actinomycete is a versatile methanol-utilizing bacterium, closely related to *Amycolatopsis mediterranei* and *Saccharopolyspora erythraea* (12, 16). Here, we report an analysis of the pMEA300 segment involved in site-specific integration.

Growth, DNA isolation, manipulation, and sequencing. Growth of *A. methanolica* was on complete medium containing (per liter) yeast extract (BBL) (4 g), malt extract (Difco) (10 g), and glucose (4 g). Plasmid DNA of *A. methanolica* was isolated by using the hot alkaline lysis method (14). Isolation of total cellular DNA was performed as described previously (13). Transfer of DNA fragments to Hybond-N nylon membranes (Amersham, Amersham, U.K.) was performed as described previously (24). DNA sequencing was performed with the Automated Laser Fluorescent DNA Sequencer from Pharmacia LKB. Sequencing reactions were done by the dideoxy method (23) with T7 polymerase and with either 5′-end-labelled primers (30) or with unlabelled primers and fluorescein-labelled ATP (27). The nucleotide sequence data were analyzed using the programs supplied in the PC/GENE software package (IntelliGenetics, Mountain View, Calif.). Open reading frames were identified by using a *Streptomyces* codon usage table (28). Nucleotide and deduced amino acid sequences of the identified open reading frames were compared with sequences in the database by using the Blast program (1). The nucleotide sequence data determined in this report has been deposited in the GenBank database under the accession number L36679.

Detection of free and integrated pMEA300 and location of the *attP* site. Covalently closed circular DNA could be isolated from CsCl-ethidium bromide gradients containing *A. methanolica* cell lysates. Restriction analysis of this plasmid, designated pMEA300 (Fig. 1A), revealed a size of 13.3 kb. The possible presence of an integrated form of pMEA300 in *A. methanolica* was examined by Southern hybridization using

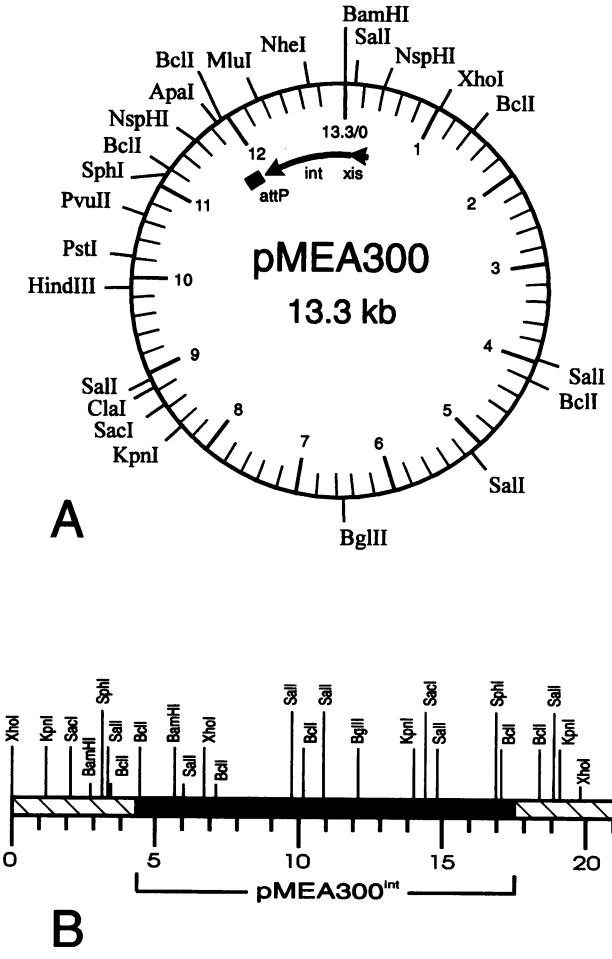

FIG. 1. Restriction maps of free (A) and integrated (B) forms of pMEA300 (the scale is in kilobases). No restriction sites were found for *Eco*RI, *Eco*RV, *Nde*I, or *Xba*I.

* Corresponding author. Phone: 31.50.632150. Fax: 31.50.632154. Electronic mail address: L.Dijkhuizen@biol.rug.nl.

† Present address: Department of Internal Medicine, Medical Clinic B, University Hospital, Remistrasse 100, CH-8091 Zürich, Switzerland.

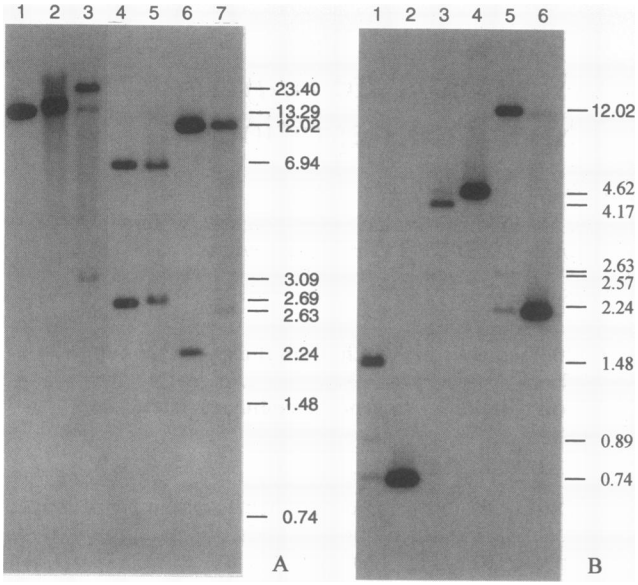

FIG. 2. Southern hybridizations of ^{32}P-labelled pMEA300 (A) or the 0.74-kb *Bcl*I pMEA300 fragment (B) with restriction digests of total DNA of *A. methanolica* (panel A, lanes 2, 3, 5, 7, and panel B, lanes 1, 3, and 5) and pMEA300 DNA (panel A, lanes 1, 4, and 6, and panel B, lanes 2, 4, and 6). The following restriction enzymes were used: panel A, *Sph*I (lanes 1 and 2), *Bam*HI (lane 3), *Bcl*I (lanes 4 and 5), and *Bam*HI + *Sph*I double digestion (lanes 6 and 7); panel B, *Bcl*I (lanes 1 and 2), *Sal*I (lanes 3 and 4), and *Bam*HI + *Sph*I double digestion (lanes 5 and 6). The lambda *Hin*dIII fragments were used as size standards. The sizes of the hybridizing bands are indicated on the right in kilobases.

*Bam*HI, *Sph*I, and *Bcl*I digests of total DNA (Fig. 2A). This revealed that pMEA300 was present as part of a larger replicon. Apparently, pMEA300 integration occurs via recombination in the 0.74-kb *Bcl*I pMEA300 fragment, thus carrying the *attP* site. Confirmation for this was obtained in a second hybridization experiment using the 0.74-kb *Bcl*I fragment as a probe (Fig. 2B). From the difference in hybridization intensity we estimated that the position of the integration site was 0.64 kb from the *Sph*I-*Bcl*I sites (Fig. 1A). The data from Fig. 2 and similar experiments allowed the construction of a restriction map of the integrated plasmid (pMEA300^{int}) and its flanking regions (Fig. 1B). The restriction map of the integrated copy was found to be identical to that of the free copy. Unlike the situation for pMEA100 in *A. mediterranei* (29), rearrangements and amplifications of pMEA300 DNA fragments were not detected.

Nucleotide sequence of the *Nsp*HI pMEA300 segment involved in site-specific integration. The nucleotide sequence of the *Nsp*HI fragment (2,100 bp) was determined (Fig. 3). The G+C content was 68.6%. Two complete open reading frames, designated *int* and *xis* for reasons explained below, were

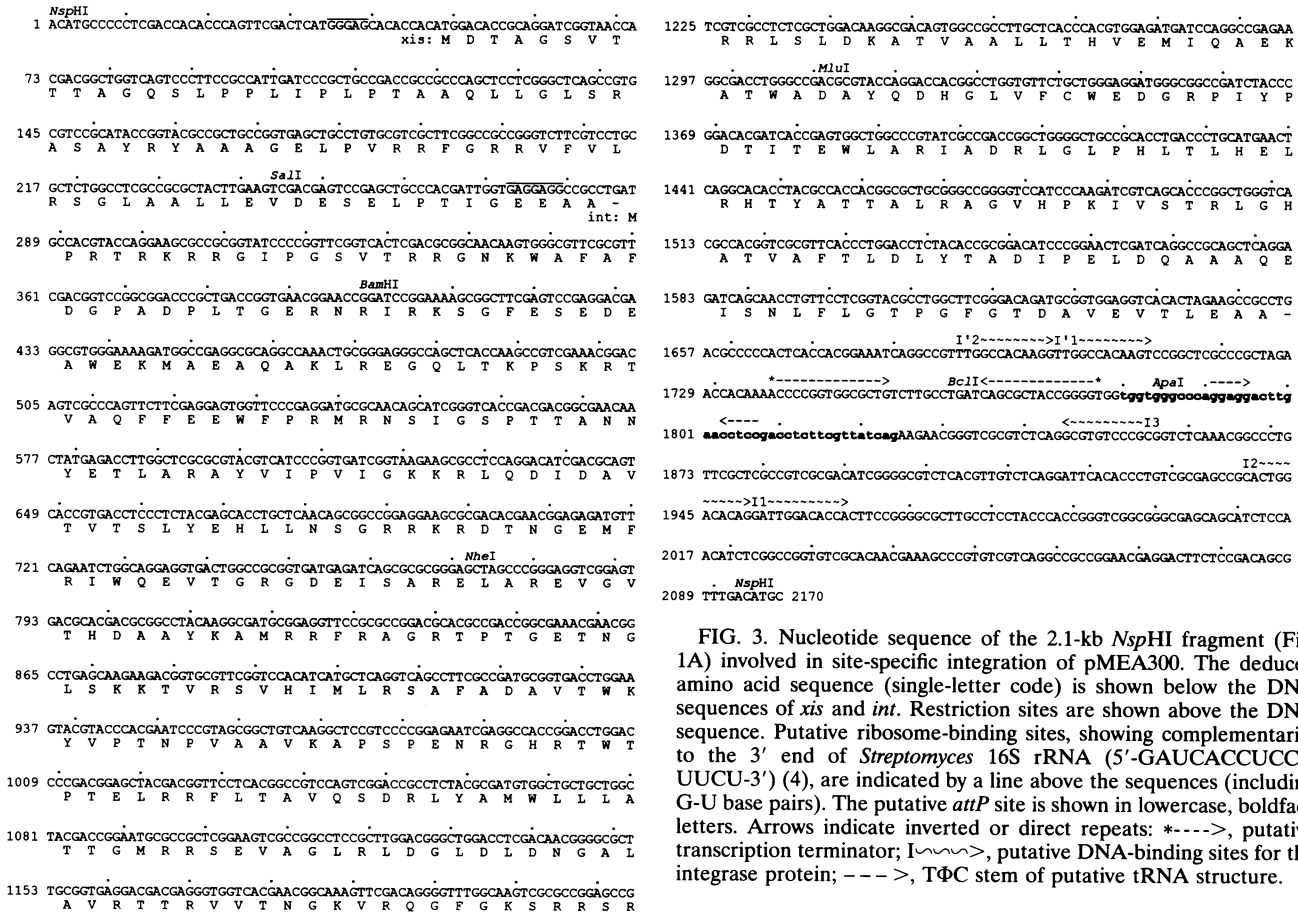

FIG. 3. Nucleotide sequence of the 2.1-kb *Nsp*HI fragment (Fig. 1A) involved in site-specific integration of pMEA300. The deduced amino acid sequence (single-letter code) is shown below the DNA sequences of *xis* and *int*. Restriction sites are shown above the DNA sequence. Putative ribosome-binding sites, showing complementarity to the 3′ end of *Streptomyces* 16S rRNA (5′-GAUCACCUCCU UUCU-3′) (4), are indicated by a line above the sequences (including G-U base pairs). The putative *attP* site is shown in lowercase, boldface letters. Arrows indicate inverted or direct repeats: *---->, putative transcription terminator; I∿∿∿>, putative DNA-binding sites for the integrase protein; – – – >, TΦC stem of putative tRNA structure.

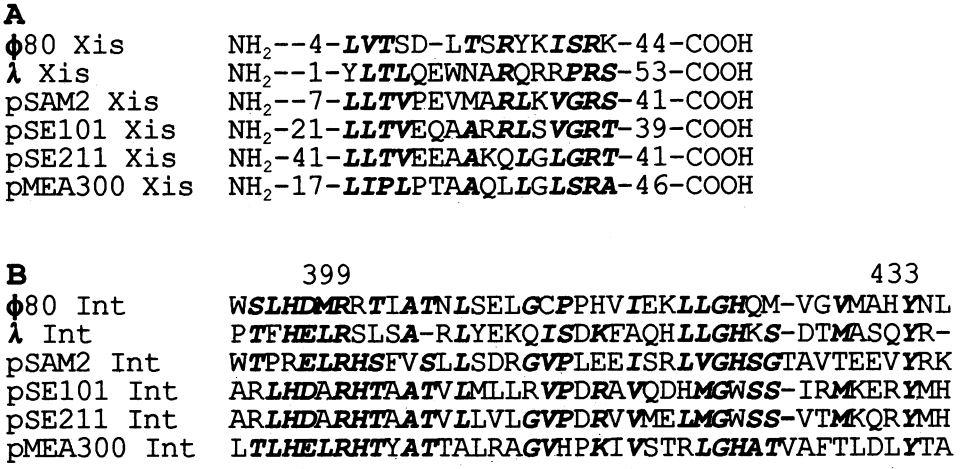

FIG. 4. Amino acid sequence alignments of Xis (A) and Int (B) of pMEA300 with the conserved segments of the Xis and Int proteins of bacteriophages λ and Φ80 (2) and with the putative Xis and Int proteins of plasmids pSAM2 (7), pSE101 (9), and pSE211 (10). Identical or conserved amino acids at a given position (a minimum of four identical or conserved) are shown in italic, boldface type according to the following conservation scheme: ILMV, ASTPG, DE, RK, NQ, FYW, C, and H.

predicted. The deduced amino acid sequences of these open reading frames are shown in Fig. 3. *xis* starts at ATG-48 and terminates at TGA-285 (Fig. 3), encoding a relatively small (79 amino acids; M_r, 8,239) and basic protein. Xis displays considerable similarities with Xis proteins of the plasmids pSAM2, pSE211, and pSE101 (Fig. 4A). The first two possible start codons (ATG-3 and ATG-33) of *xis* are not preceded by recognizable ribosome-binding sites. Only the third methionine (ATG-48) of this open reading frame is preceded by a good ribosome-binding site (GGGAG).

Starting at ATG-287 and terminating at TGA-1655, *int* is postulated to encode a protein of 456 amino acid residues (M_r, 51,134). The start codon of *int* is preceded by a potential ribosome-binding site (GAGGAGG). Int shares extensive similarity with the C termini of proteins belonging to the Int family of site-specific recombinases (Fig. 4B). Residues His-396, Arg-399, and Tyr-433 (family positions; Fig. 4B) are conserved in all sequences (except for Int of pSAM2) and are present as His-383, Arg-386, and Tyr-419 in the Int of pMEA300. It thus appears very likely that *int* encodes the pMEA300 integrase. Deletion of the *Bam*HI-*Apa*I region of pMEA300, which lies within the coding region of the *int* gene, indeed results in nonintegrating, autonomously replicating plasmids (27a). Five imperfect repeats were found immediately upstream of *int* and to the right of *attP* (Fig. 3; see below). These repeats could serve as possible DNA-binding sites for the Int protein, as has been suggested for pSAM2 (7). Similar consensus sequences can be derived for these repeats at similar positions on pMEA300 and pSAM2.

Analysis of the *attP* region. Current evidence indicates that *attB* sites are contained within tRNA genes (3, 6, 9, 22, 26). A database screen revealed that nucleotides 1780 through 1824, located downstream of *int* (Fig. 3), show extensive similarity to known *attB* sites (not shown) (9) and especially to tRNA sequences from various organisms. The highest score, of the pMEA300 *attP* (shown below in inverse complement), was obtained with an Ile-tRNA gene of *Rhodobacter sphaeroides* (X53853:RSRRNA).

A.m. 1824-CT**GAT**AACGAAGAGGTCGGAGGTTCAAGTCCTCCTGGGCCCACCA-1780
R.s. 2227-CT**GAT**AAGCGTGAGGTCGGAGGTTCAAGTCCTCCTCGACCCACCA-2271

This indicates that *attP* of pMEA300 is approximately 44 bp long and that chromosomal integration occurs within an Ile-tRNA gene with an ATC anticodon (shown in boldface type).

In future work we will study the other pMEA300-encoded functions, using a pMEA300-negative derivative strain of *A. methanolica* (27a). It is expected that this information, and a clear understanding of the regulation of pMEA300 integration and excision in relation to growth conditions, will provide further insights into the physiological role of this type of plasmid.

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